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Acute and latent adenovirus in COPD

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Summary

Introduction: The COPD airway is infiltrated with CD8+ T cells, which has led to a virus being implicated in its pathogenesis. Some investigators have suggested a role for the persistence of the adenovirus E1A in bronchial epithelial cells. We examined respiratory tract specimens from COPD patients for the presence of E1A DNA and mRNA using real-time PCR.

Methods: Nucleic acid extraction was performed on sputum specimens from patients with COPD. Copy numbers for GAPDH, and adenovirus 5 E1A DNA and mRNA were determined using a quantitative real-time PCR assay. All samples were screened for the adenovirus hexon gene using nested PCR.

Results: One hundred and seventy-one patients, 80 male, aged 68.9 ± 9.8 years with COPD were recruited. One hundred and thirty-six were seen during an exacerbation when admitted to hospital, 33 of whom were reviewed when clinically stable along with an additional 35 stable COPD patients. Ten patients in the exacerbation group were positive for the adenovirus hexon gene (7%), as were four in the stable group (6%). Only two patients in the exacerbation group were positive for adenovirus 5 E1A. Only one patient in the stable COPD group had detectable E1A DNA/mRNA and also tested positive for the adenovirus hexon gene.

Conclusion: Adenovirus is detected in similar frequencies in exacerbated and stable COPD patients. Adenovirus E1A DNA is infrequently detected in respiratory secretions from patients with COPD. Our data suggest that the persistence of adenovirus 5 E1A in lung cells of sputum samples in patients with COPD occurs infrequently.

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Introduction

COPD is a progressive disease which progresses over the lifetime of the patient.¹ It is currently the sixth commonest cause of death, and it is due to rise to the third commonest cause by 2020.² Only 20% of smokers develop airflow obstruction.^{1,3} It has thus been postulated that other factors including latent adenoviral infection may amplify the inflammatory response to cigarette smoke resulting in the COPD phenotype.⁴ Previous work investigating the role of latent adenoviral infection has focused on group C adenovirus, adenovirus 5 early expressed antigen (E1A).⁵ Group C adenovirus is endemic and children are often infected resulting in latent infection.⁴ The development of COPD is associated with poverty,⁶ and infections are more likely to develop in children of lower socioeconomic status.⁷

Adenoviruses are nonenveloped, double-stranded DNA viruses, and over 50 serotypes have been isolated and characterized into 6 subgroups (A–F). Types 4 (group E), 7 (group B) and 1, 2 and 5 (group C) viruses most frequently cause respiratory disease in adults. The incubation period is 5–8 days. Infections usually occur within the first year of life with most children having serological evidence of infection by 6 months.⁸ It is recognized as a common respiratory pathogen in children but is infrequently detected in adult populations with COPD with detection in 0.5–1.5% of exacerbations.^{9,10} Detection of adenovirus in bronchoalveolar lavage fluid (BALF) has been linked to severe lower respiratory tract infection in immunocompromised individuals.¹¹ Adenovirus has also been linked to more symptomatic episodes and even near fatal asthma.¹² Mitchell et al.¹³ showed that adenoviral infection is significantly under reported when using non-molecular methods for detection. These findings suggest that adenoviral infection may be more common in immunocompromised individuals. Also asymptomatic illness can be followed by latent viral infection.

Exacerbations of COPD account for significant morbidity and mortality.^{14,15} Bacteria are isolated from sputum in 40–60% of acute COPD exacerbations.^{16–18} Some COPD patients suffer from frequent exacerbations. This subgroup have a worse health status and a more rapid decline in FEV₁.^{19,20} Studies using serology and viral culture identified respiratory viruses in 30% of patients during acute exacerbations of COPD.²¹ Recently with the development of more sensitive molecular tests the role of viruses in COPD has been better defined.^{9,10,22}

As detailed above latent adenoviral infection is proposed as a causative factor in the pathogenesis of COPD. We wished to determine the prevalence of acute (adenovirus hexon gene) and latent adenoviral infection (E1A gene) in COPD exacerbations and also to determine if there was latency of this virus by testing stable COPD patients.

Methods

Subjects

One hundred and thirty-six patients hospitalized with COPD were recruited within 24 h of presentation. Thirty-three of these patients were subsequently reviewed when clinically

stable. A further 35 stable COPD patients were recruited (Fig. 1). All recruitment was carried out over a 2-year period. Stability was defined as no change in respiratory symptoms in the previous 8 weeks. Spirometry and reversibility (improvement in FEV₁ following nebulised beta agonist) were performed. The best of 3 reproducible readings was taken. The test was performed with a Vitalograph spirometer (Vitalograph, Buckingham, England). Reversibility was performed after nebulised beta agonist (salbutamol 2.5 mg). Exacerbated and stable COPD subjects were included in this study in order to determine if E1A detection was related to acute adenoviral infection whilst also to find if patients remained E1A positive or negative by sampling at two time points. This study was approved by the Queen's University Belfast ethics committee and all patients gave written consent.

Inclusion/exclusion criteria and definitions

COPD diagnosis and assessment of severity was classified according to the GOLD criteria.²³ Exacerbation of COPD were defined using inclusion criteria as detailed above with symptoms of increased dyspnoea, increased sputum purulence or increased sputum production.²⁴ Stable COPD was defined using inclusion criteria as detailed above without any symptoms of exacerbation or changes in treatment within the last 8 weeks. Those patients with a history of bronchiectasis, neoplastic process or other serious concomitant disease were excluded. Patients who had significant improvement following bronchodilator therapy (+200 ml in FEV₁/+15%) were excluded.

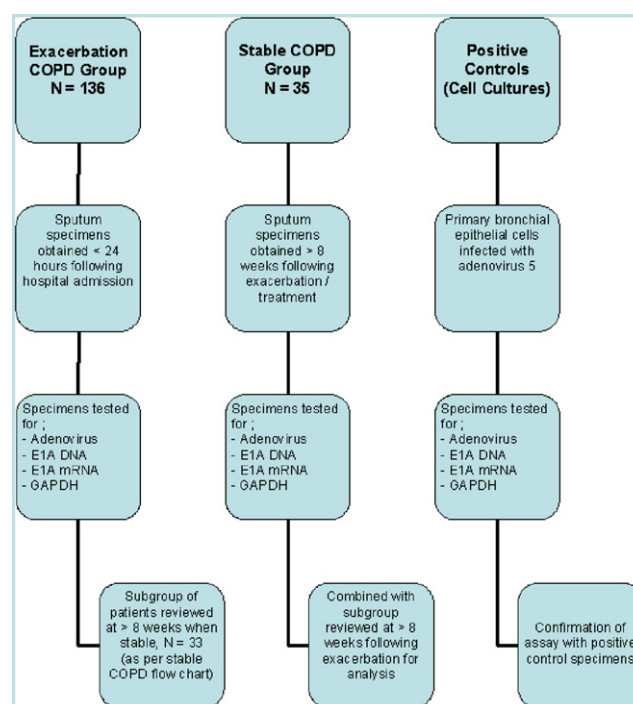


Figure 1 Study design.

Power calculation

This study was linked to a COPD exacerbation study examining the role of acute respiratory viral infection in exacerbations of COPD (unpublished data). Assuming an acute respiratory virus detection rate of 30% amongst COPD patients during exacerbations and 10% detection when stable a power calculation was performed using EpiInfo™ version 6. This calculation was performed in order to determine group size required to detect a significant difference in the frequency of respiratory virus infection between exacerbated and stable COPD groups. Using a confidence value of 95% with a power of 80% and a 2:1 ratio of group sizes, 111 patients with COPD exacerbations and 56 stable COPD subjects were required to detect a significant difference in the incidence of respiratory virus infection between groups.

Samples

Sputum sampling

Sputum samples were obtained either by spontaneous production or following nebulised hypertonic saline. Briefly, 4 ml of 3% saline was nebulised via an air driven nebuliser (Micro Mist® small volume nebuliser, Hudson Respiratory Care Inc., Leicestershire, UK). Every 5 min spirometry was repeated to measure FEV₁, and nebulization continued if FEV₁ had not fallen by more than 20%. This was continued up to 20 min. All sputum expectorated was collected.

Sputum processing

All samples were processed within 2 h. Specimens were mixed with 4 volumes 0.1% dithiothreitol (Sigma, Poole, UK) and shaken in an orbital incubator (Gallenkamp, Loughborough, UK) for 15 min at 37 °C followed by the addition of 4 volumes phosphate buffered saline and shaken for a further 5 min. The resulting suspension was then filtered through 50 µm Nylon Gauze (Lockertex, Warrington, UK) and spun down at 1000g for 10 min. After removing the supernatant the cell pellet was resuspended in Lysis Buffer (Qiagen, Crawley, UK). Total nucleic acid extraction was performed on 200 µl of sputum sample suspended in Lysis Buffer (QIAamp DNA Blood Mini Kit).

Polymerase chain reaction

Following nucleic acid extraction, 2 µl of each specimen was combined with 8 µl of mastermix containing primers specific to adenovirus 5 E1A DNA or mRNA. All primers and probes are shown in Table S1. First round mastermix was prepared using the Access RT PCR System (Promega, Southampton, UK). PCR was performed on a Peltier thermal cycler (MJ Research, Essex, UK) for 20 cycles and 0.2 µl of the first round product was then used in combination with 9.8 µl of mastermix containing primers and a TaqMan® (Qiagen, Crawley, UK) probe (labeled with FAM and TAMRA dyes) internal to the first round primers. Real-time PCR (30 cycles) was carried out using a Roche LightCycler (Roche Diagnostics Corporation, Mannheim, Germany) with log dilutions of the cloned target sequence being used as calibrators thus enabling quantitation of copy numbers in unknown speci-

mens (Fig. 2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also employed, using identical methods to those above, as a housekeeping gene, and using a published primer and probe set with a set of external nested primers.²⁵

The E1A DNA primers used in these experiments were designed internal to those described in previous E1A work.²⁶ Nested PCR primers and a probe were also designed for adenovirus E1A mRNA using published sequences and DNASTar primer design software (DNASTar, Madison, USA). The E1A DNA, E1A mRNA and GAPDH LightCycler assays of the cloned target sequences had detection limits of 5, 7 and 6 copies per reaction, respectively, added in a 2 µl volume (in contrast to the LightCycler PCR reaction volume which was 10 µl). Coefficient of variation for these assays was calculated at between 5% and 13%. A positive result was recorded if a copy number greater than zero was obtained following completion of real-time PCR.

All specimens were tested for the adenovirus hexon gene (capsular protein) using a nested PCR approach and published primers,²⁷ the technique for this assay has previously been described.¹³ The hexon PCR primers are designed to detect up to 18 subtypes of adenovirus, including the respiratory subtypes B–E. Results were obtained using gel electrophoresis. A positive result was recorded if there was a band seen corresponding to the correct size for a first or second round product (Fig. 3). Further details of all of the above assays are listed in Appendix A1.

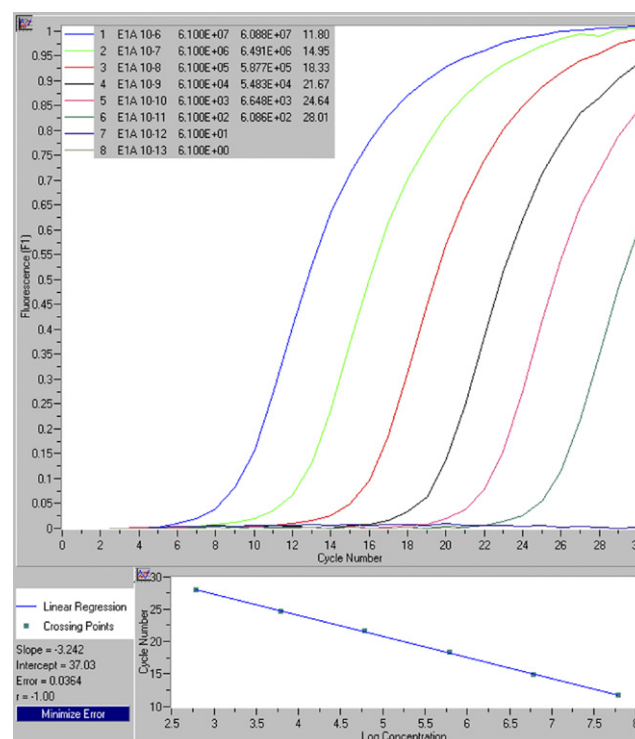


Figure 2 Log dilutions of E1A cloned material run on a Lightcycler. Notation: This illustrates log dilutions of Adenovirus E1A DNA cloned target (10^{-6} – 10^{-13}) which were run on the Lightcycler to perform an end point titration. Second round samples were PCR positive out to 10^{-11} dilution.

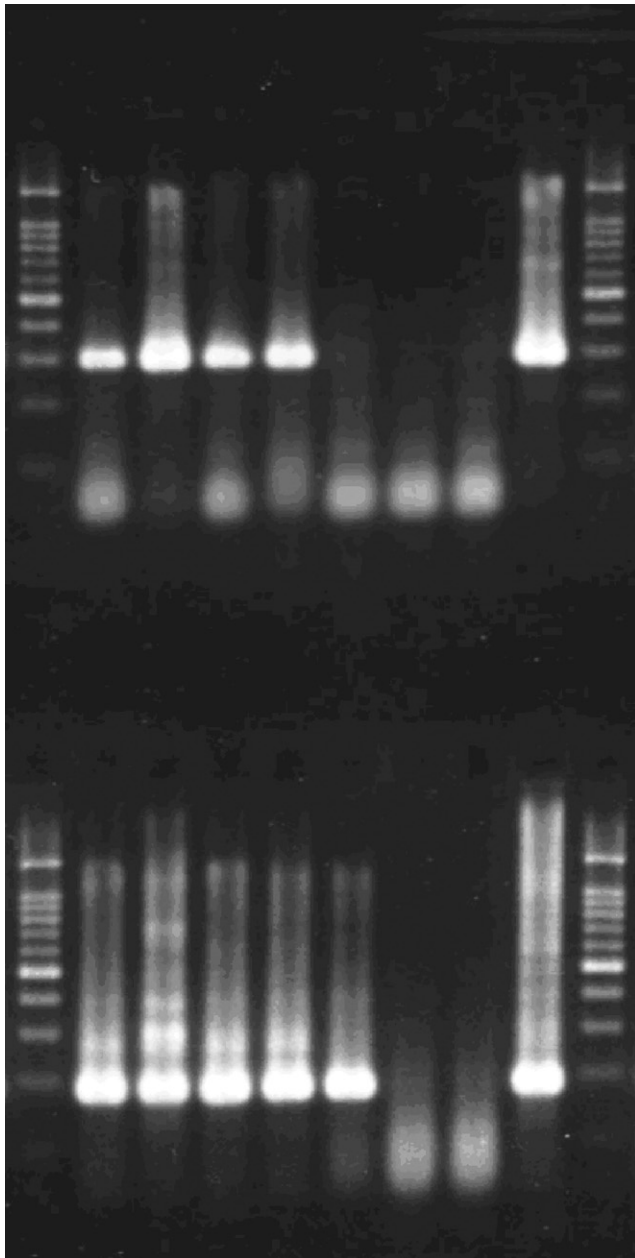


Figure 3 Testing clinical specimens for adenovirus hexon gene. Notation: Eight clinical specimens tested for adenovirus using nested primers. Molecular weight markers flank the PCR products. Top and bottom products represent first and second round results, respectively. Positive control is in lane 8. Specific first and second round product sizes of 301 and 185 base pairs are seen.

Cell culture inoculation

We infected primary bronchial epithelial cells with adenovirus 5 as positive test specimens. Primary cultures of bronchial epithelial cells obtained by non-bronchoscopic brush method were cultured as previously described.²⁸ Nucleic acid extraction and PCR techniques were identical to those detailed above. This study was approved by the Queen's University Belfast ethics committee and written consent obtained. Further details are listed in Appendix A1.

Results

COPD patients

One hundred and seventy-one patients, 80 male, aged 68.9 ± 9.8 years (mean \pm SD) with COPD, FEV₁ 0.90 ± 0.52 l, 41% predicted, were recruited. Ninety-two percent of patients had smoked 46.1 ± 36.3 pack years (Table 1). One hundred and thirty-six were seen during an exacerbation when admitted to hospital, 33 of whom were reviewed when clinically stable along with an additional 35 stable COPD patients. Real time PCR analysis for GAPDH mRNA showed a median (IQR) copy number of 2295 (365–12,101) copies per epithelial cell in the specimens (Table 2). Ten exacerbated patients were positive for the adenovirus hexon gene (capsular protein). Only two patients in the exacerbation group were positive for adenovirus 5 E1A DNA with a copy number of 3.4×10^5 and 7.0×10^4 copies per epithelial cell. Nine of the patients testing positive for the adenovirus hexon gene were negative for E1A, the remaining one patient who had tested positive for both the adenovirus hexon gene and E1A was negative on repeat testing for E1A (DNA and mRNA) and the adenovirus hexon gene when retested 3 months later. Only one patient in the stable COPD group had detectable E1A DNA/mRNA 3003/1257 copies per epithelial cell, this patient initially tested negative for adenovirus, E1A DNA and E1A mRNA. It was also noted that this patient plus one other patient in this stable group tested positive for the adenovirus hexon gene.

Epithelial cell cultures (positive controls)

Eleven epithelial cell cultures were inoculated with adenovirus 5. In each case higher copy numbers of adenovirus were detected following infection than were present in the inoculating dose. Cytopathic effect was seen in each case. GAPDH was detectable in all specimens; median (IQR) 2.4×10^7 (1.6×10^6 – 5.0×10^7) copies per reaction. All samples tested positive for the adenovirus hexon gene. Adenovirus 5 E1A DNA was detectable in all samples; 1.7×10^{12} (8.3×10^{10} – 3.8×10^{12}) copies per reaction, or when adjusted for GAPDH; 4.2×10^4 (1380 – 2.6×10^5) copies per reaction. Adenovirus E1A mRNA was also detectable in all samples; 8.0×10^{10} (2.7×10^{10} – 1.2×10^{12}) copies per reaction, or when adjusted for GAPDH; 22773 (446 – 6.2×10^4) copies per reaction. Adenovirus hexon gene and E1A DNA/mRNA were detected at all time points (6, 12, 24, 48, 72 h).

Discussion

Adenovirus infection occurs in 7% of exacerbations, however adenovirus 5 E1A was found in only two patients with exacerbations of COPD. One of these two patients who had detectable E1A and capsular gene, these were not present 3 months later, indicating an acute adenoviral infection. This finding suggests that the initial positive E1A result was related to an acute adenovirus infection, as the same patient was negative for both on subsequent screening. Only one patient in the stable COPD group had detectable E1A and also tested positive for the adenovirus hexon gene.

Table 1 Patient demographics.

Measurement	Patient group		
	Exacerbated COPD	Stable COPD (reviewed)	Stable COPD
No. of patients	136	33	35
Sex (M/F)	64/72	14/19	16/19
Age	70 ± 9.4	69 ± 8.4	64 ± 9.7
FEV ₁ -litres (% Pred.)	0.84 ± 0.47 (39 ± 20)	0.90 ± 0.4 (48 ± 23)	0.98 ± 0.5 (48 ± 22)
Smoking (Pk/Yr)	48.0 ± 39.2	45.9 ± 29.7	38.7 ± 21.8

Table 2 Cell count and PCR assay results.

Measurement	Patient group		
	Exacerbated COPD	Stable COPD (reviewed)	Stable COPD
No. of patients	136	33	35
Adenovirus	10/136	2/33	2/35
Hexon gene (%)	7	6	6
E1A DNA	2/136	1/33	0/35
Positive (%)	1.5	3	0
E1A DNA	(1) 341,500 ^{*,†}	3003 [*]	0
Copy no./cell [‡]	(2) 1,597,413		
E1A mRNA	2/136	1/33	0/35
Positive (%)	1.5	3	0
E1A mRNA	(1) 70,100 ^{*,†}	1257 [*]	0
Copy no./cell [‡]	(2) 130		
Epithelial cell count (× 10 ⁴ /mm ³)	1.0 (0.13–3.81)	2.63 (0.82–8.0)	2.69 (0.91–5.84)
GAPDH	6755	1548	82
Copy no./cell	(1879–40,370)	(337–3933)	(24–275)

*Positive on testing for the adenovirus hexon gene.

†Negative for both Adenovirus hexon gene and E1A (DNA & mRNA) on repeat testing 3 months later.

‡Corrected to epithelial cell count. (1) and (2) identify two individual patients.

These findings suggest that in two cases the initial positive E1A result was related to an acute adenoviral infection. In one patient in the exacerbation group in whom E1A DNA and mRNA were detected in the absence of the hexon gene is a potential representative of a patient with a latent infection. The detection of latent adenoviral infection is lower than has previously been reported,⁵ potential reasons are discussed later in this paper. The detection of capsular gene in the absence of adenovirus 5 E1A occurs in infection with adenovirus which is not adenovirus 5. There are over 50 different serotypes of adenovirus which have been isolated and characterized into 6 subgroups (A–F). Ten patients in the exacerbated group and a total of four in the stable group tested positive for the adenovirus hexon gene. As detailed above only two of these patients also had detectable E1A implying that in the remainder of cases the infection was with an adenovirus other than adenovirus 5. The detection rate of adenovirus in exacerbations of COPD is higher in this study than has been previously reported.^{9,10} This may be in part due to increased sensitivity of using a nested PCR technique or alternatively a coincidental clustering of cases during the recruitment phase of this study. This study

included two adenovirus E1A assays which were directed at detecting adenovirus E1A DNA and a separate assay to measure adenovirus E1A mRNA. Thus each specimen was tested twice for different regions of the E1A sequence.

This study differed from others in that we examined respiratory secretions whereas previous investigators looked at lung tissue.⁵ However, the hypothesis that has been put forward is that E1A is located in the epithelial cell.⁵ The airway is lined extensively with epithelial cells which are shed in sputum. In this study there were a median of 1×10^4 or more epithelial cells per ml of specimen and we utilized assays which had a detection limit of less than 5000 copies per ml. Published experimental work used E1A positive control specimens consisting of Graham 293 cells transformed with 4–5 copies of the E1A region of adenovirus 5.⁵ There were similar levels of detection in positive control samples as compared to clinical specimens. Thus E1A, if present, would be detectable in the sputum specimens analysed in this study using the real-time E1A assay developed. However the above reasoning is based on E1A residing predominantly within epithelial cells, if this were incorrect lack of detection may be a reflection of the sample

used rather than actually reflecting a true negative result. Also there is the possibility that viral latency or persistence will not necessarily result in epithelial cell lysis, and thereby lung epithelial cells harboring viral nucleic acid might not be shed into sputum.

As a positive control study we infected primary bronchial epithelial cells with adenovirus 5. All cultures became infected and demonstrated shedding of higher copy numbers of viruses than were contained in the initial inoculum (data not shown). In each case high copy numbers of E1A were detectable, confirming its role in adenovirus infection. E1A was found in all positive control samples of adenovirus infected cultured epithelial cells which were sampled at different time points post inoculation (6, 12, 24, 48, 72 h), illustrating that E1A was present over this range of sampling time points. A strength of this study is that the controls were not obtained from COPD patients and as such this factor avoided any possibility of E1A infection of COPD control samples acting as a confounding variable. A possible source of error was that the relatively low numbers of epithelial cells may result in E1A not being detected because it thought to be present in these cells only. Although the positive control specimens were not processed in the same way as sputum specimens (i.e. using DTT) we still identified the adenovirus hexon gene and E1A using PCR assays. Sputum is known to contain PCR inhibitors, some authors have suggested dilution of samples in an attempt to remove these inhibitors, sample dilution was not performed in this study and could be contested as a reason for reduced detection of E1A.²⁹ However GAPDH was detected in all specimens and this result would preclude the possibility that PCR inhibitors were the cause of the E1A negativity. The findings of the present study should be confirmed with analysis of lung tissue specimens in order to be directly comparable with the initial findings in this area. The possibility of persistent or latent viral infection playing a role in the pathogenesis of COPD however remains an attractive hypothesis. The predominance of a cytotoxic T cell infiltrate response in the airway mucosa of COPD patients would fit with a viral infection.

Conclusions

In summary, this study has shown that adenovirus can infect bronchial epithelial cells and that E1A is detectable in experimental infection. Adenovirus was sometimes found in COPD patients during exacerbation and when stable. Adenovirus E1A DNA is rarely detected in sputum from patients during exacerbations of COPD using a validated and sensitive assay. Our data suggest that the persistence of adenovirus 5 E1A in lung cells of sputum samples in patients with COPD occurs infrequently

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Anne Bingham and Dr. Gary Doherty (Department of Clinical Biochemistry) sampled, grew and infected the PBEC lines with adenovirus 5.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at: [doi:10.1016/j.rmed.2007.05.015](https://doi.org/10.1016/j.rmed.2007.05.015).

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